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Identifying Human Herpesvirus 8 Infection: Performance Characteristics of Serologic Assays

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Summary: Epidemiologic studies of infection with the oncogenic human herpesvirus 8 (HHV-8) depend on serologic methods to diagnose infection. However, optimal strategies for identifying HHV-8 infection remain undefined. We therefore evaluated four enzyme-linked immunoassays (EIAs) and one immunofluorescence assay (IFA) using sera from 87 individuals with the prototype HHV-8 disease, Kaposi's sarcoma (KS), and 210 participants in a hemophilia study (who were presumed not to be infected with HHV-8). Assays performed reasonably well in distinguishing between infected and uninfected persons, with receiver operator curve areas between 0.86 and 0.96. Nonetheless, IFA had only 86% sensitivity and 88% specificity, and no EIA simultaneously had sensitivity and specificity above 90% for any of the optical density (OD) cutpoints used to define seropositivity. Some assays were markedly less sensitive with diluted KS sera, suggesting that they poorly identify low-titer antibodies present in asymptomatic infection. We also developed a classification tree that categorized individuals as seropositive if they had OD > 2.00 on recombinant K8.1 protein EIA or if they had both K8.1 OD between 0.51 and 2.00 and positive IFA results; this strategy had between 80% and 90% sensitivity and 95% and 100% specificity. Overall, assays performed adequately for use in most epidemiologic investigations, but wider applications will require improved tests. **Key Words:** Human herpesvirus 8—Kaposi's sarcoma—Diagnostic tests—Receiver operator curves—Serology—Sensitivity—Specificity.

The recently discovered human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma (KS) herpesvirus, is essential in KS pathogenesis. HHV-8 DNA is found in all types of KS tumors (1), including those associated with AIDS and those in elderly adults in Mediterranean countries (classical KS).

Some epidemiologic studies of HHV-8 infection have used serologic methods to identify infection, because a large proportion of infected individuals do not have detectable HHV-8 DNA in peripheral blood (2,3). However, there remain concerns regarding the accuracy of HHV-8 serologic assays. Individual tests have imperfect sensitivity. For example, antibodies against proteins expressed during latent HHV-8 infection are detectable by indirect immunofluorescence assay (IFA) in 80% to 95% of KS patients (4–8). Antibodies to lytic phase proteins, such as recombinant orf65 capsid protein or whole virus, as measured by enzyme-linked immunoassays (EIAs), appear in similar proportions of KS patients (4,6,9). More important, it remains unknown how well these assays identify asymptomatic HHV-8-infected individuals, who have lower antibody levels than KS patients (5,6).

The specificity of serologic tests also is uncertain because few studies have systematically evaluated groups

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who are clearly at low risk for HHV-8 infection. Seroprevalence among volunteer blood donors in the United States and United Kingdom has been from 0% to 10% in most studies (4,6–8), suggesting that infection is uncommon in these populations and that the serologic tests are highly specific. However, test sensitivity and specificity depend on the cutpoint chosen to define a positive result. As the cutpoint is lowered, sensitivity increases and specificity decreases (10), so that serologic tests that correctly identify a large proportion of individuals with KS might also have low specificity. This inherent tradeoff has received little attention (11).

A better understanding of HHV-8 serologic tests might help to interpret epidemiologic data and identify improved test strategies. We therefore systematically evaluated several serologic tests (an IFA and four EIAs) using a panel of reference samples. To measure specificity, we tested men with hemophilia and their female partners living in the United States. HHV-8 infection in this population is likely rare, because KS is uncommon in AIDS patients with hemophilia in the United States (12), despite a 100,000-fold increase in KS risk associated with AIDS (13). To measure sensitivity, we used samples from individuals with AIDS-associated and classical KS. In addition, we quantified the ability of serologic tests to detect lower-titer HHV-8 antibodies by analyzing diluted samples from KS-affected study subjects. We varied cutpoints for these assays to examine the tradeoff between sensitivity and specificity. Finally, we evaluated combinations of tests to determine whether we could simultaneously increase sensitivity and specificity.

METHODS

Study Subjects

We studied 87 individuals with KS: 51 men with AIDS-associated KS (mean age 39 years) and 36 Sardinian patients with classical KS (72% male, mean age 71 years). We also studied 210 well-characterized subjects enrolled in a longitudinal study of hemophilia (14): 134 were men with hemophilia (mean age 39 years; 88% HIV-infected) and 76 were female sexual partners of men with hemophilia (mean age 35 years; 12% HIV-infected). The men with hemophilia and their partners (hereafter referred to as hemophilia study subjects) were from the United States and did not have KS before or at the time of HHV-8 testing or during a total of 373 person-years of subsequent follow-up (median follow-up 0.9 years).

Biologic Specimens and Serologic Assays

Specimens (90 serum, 207 plasma) were stored at −70°C until testing. Antibodies to HHV-8 were measured in these specimens using five different serologic assays. Two assays, an IFA and a recombinant protein EIA, measured antibodies to the latent nuclear antigen (LANA or LNA-1) encoded by orf73. Two recombinant protein EIAs were used to measure antibodies to the minor capsid protein orf65 and the lytic phase glycoprotein K8.1. The fifth assay, also an EIA, used whole HHV-8 virions (Advanced Biotechnologies Inc., Columbia, MD, U.S.A.). All assays were performed by laboratory personnel blinded to study subjects' KS status, and the IFA was interpreted without knowledge of EIA results.

The IFA was performed using the latently HHV-8-infected primary effusion lymphoma cell line BCP-1 (15), with the HHV-8-negative Ramos' cell line (16) as a negative control. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton × 100 at room temperature. Serum or plasma samples were diluted 1:100 in phosphate-buffered saline containing 3% fetal bovine serum (PBS/FBS) and incubated at room temperature for 30 minutes. The slides were washed three times in PBS/FBS before 30-minute incubation at room temperature with anti-human-IgG fluorescein isothiocyanate (FITC) conjugate diluted 1:35 (Roche Diagnostics, Indianapolis, IN, U.S.A.) and counterstain/blocking solution diluted 1:15 (ViroStat, Portland, ME, U.S.A.). The slides were washed once in PBS/FBS and four times in PBS and examined by ultraviolet microscopy. Positive samples were titrated by 12 twofold serial dilutions.

The orf65, orf73, and K8.1 EIAs were developed in our laboratory, based on similar assays described by others (6,17,18). Using 0.05M carbonate/bicarbonate buffer solution at pH 10.0, orf65 was diluted 1:400 (final concentration 1.25 μg/ml), orf73 was diluted 1:200 (final concentration 0.63 μg/ml), and K8.1 was diluted 1:1000 (final concentration 1.00 μg/ml). Orf65 and orf73 recombinant proteins were then coated onto Polysorp 96-well plates (Nalge Nunc International, Naperville, IL, U.S.A.) by adding 100 μl of diluted recombinant protein. K8.1 was coated onto Immulon4 96-well plates (Dynex Technologies, Chantilly, VA, U.S.A.) by adding 100 μl of diluted recombinant protein. The plates were covered and incubated overnight at 4°C and then washed three times with 350 μl/well of 10× wash solution (NEN Life Science Products, Boston, MA, U.S.A.). Next, 300-μl blocking solution (2.5% BSA, 2.5% normal goat serum, and 0.005% Tween 20 in PBS) was added to each well. Plates were covered, incubated for 2.5 hours at 37°C, and then washed three times with 350 μl of wash solution. Serum or plasma samples were then added to each well (100 μl diluted 1:20 in blocking solution), covered, and incubated 90 minutes at 37°C. The plates were washed five times with 350 μl/well wash solution. Then, 100-μl of goat anti-human-IgG alkaline phosphatase conjugate (Roche Diagnostics) diluted 1:3000 in blocking buffer was added to each well and plates were covered. After incubation at 37°C for 30 minutes, the plates were again washed five times. At this point, 100-μl substrate solution (pH 9.8, 10% diethanolamine, NaN_3, $MgCl\text{-}6H_2O$, 1 mg/ml para-nitrophenylphosphate) was added to each well and plates were covered. After 30 minutes at 37°C, 50-μl 3N NaOH stop solution was added to each well. The plates were read at 405 nm on an automated plate reader.

The whole virus EIA was run according to the manufacturer's specifications, except that plates were read at dual wavelengths of 450 nm and 630 nm with no blanks. We examined a range of OD values as cutpoints to define a positive result, including the manufacturer's recommended cutpoint of three times the mean OD of negative controls run on the same plate (typical negative controls had OD values below 0.05).

Statistical Analysis

For primary analyses, we considered all KS study subjects HHV-8-infected and all hemophilia study subjects HHV-8-uninfected. Thus,

we measured sensitivity as the percentage of KS subjects identified as positive and specificity as the percentage of hemophilia study subjects identified as negative. For EIAs, we varied OD cutpoints to examine the tradeoff in sensitivity and specificity, quantified as the area under the receiver operator characteristic (ROC) curve (10). ROC areas near 1 indicate that the test has good discrimination, whereas areas near 0.5 indicate that the test does not discriminate at all between infected and uninfected individuals. We calculated confidence intervals for sensitivity and specificity using the normal approximation to the binomial distribution (or the exact method when specified) and for ROC areas using published formulas (19). Additionally, we compared IFA titers between groups using Wilcoxon's rank sum test.

To determine whether combinations of EIAs performed better than individual tests, we evaluated combinations using low, medium, or high cutpoints for each EIA. Additionally, we evaluated testing strategies in which positive combinations of EIAs were confirmed by IFA (i.e., only individuals positive on EIAs *and* IFA were considered seropositive). Confirmation by IFA, considered a highly specific test, is a common testing strategy (20–22).

We also categorized individuals as seropositive or seronegative using classification trees, which are decision rules based on a sequence of splits of the study population (23). Candidate trees were constructed with a computer algorithm (*tree* in S-PLUS, Version 4.5, MathSoft, Seattle, WA, U.S.A.) using a "derivation set" of 224 randomly selected study subjects (75% of total) and various combinations of serologic tests. The algorithm generated classification trees by starting with a single root node with all study subjects in the derivation set and then splitting each node in successive iterations so that the overall classification of subjects improved. Each split was optimally chosen based on a search through each serologic test and all possible cutpoints. Nodes with fewer than 5 individuals or less than 1% of the total deviance were not split further. We simplified the better performing trees by eliminating nodes that added little to sensitivity and specificity. Finally, we selected the single tree that appeared best in the derivation set and evaluated its performance using the independent "validation set" of 73 study subjects (25% of total).

To model the ability of our assays to identify individuals with asymptomatic HHV-8 infection, who have lower levels of HHV-8 antibody than do those with KS, we performed all assays on KS subjects' samples after 4-fold and 16-fold dilutions in PBS. Because of limited serum volume, two individuals with KS were not included in this analysis.

RESULTS

Individual Tests

Using the IFA assay, 26 (12%) hemophilia study subjects had positive IFA titers (1:100 or higher), compared with 33 study subjects (92%) with classical KS and 42 (82%) with AIDS-associated KS (Figs. 1*A–C*). Among KS study subjects who were IFA-seropositive, titers were higher for study subjects with classical KS than for those with AIDS-associated KS (median 1:51200 versus 1:6400; $p = .002$), although substantial overlap was present (Fig. 1*B–C*). In contrast, among IFA-seropositive hemophilia study subjects, the median titer was 1:200 (Fig. 1*A*).

KS study subjects also had higher OD values than hemophilia study subjects for each evaluated EIA (Fig. 2*A–D*). Results with the K8.1 EIA were somewhat bimodal, but OD values of KS and hemophilia study subjects overlapped in all four EIAs, indicating that no EIA discriminated perfectly between the two groups.

Table 1 provides data on sensitivity and specificity for each evaluated test. No individual test had a sensitivity above 90% for any cutpoint evaluated in Table 1, but for each EIA, specificity was above 95% for at least one cutpoint. As OD cutpoints were raised, sensitivity decreased and specificity increased. For example, for the K8.1 EIA, for OD cutpoints of 0.80, 1.00, and 1.50, sensitivity estimates were 90%, 85%, and 78%, respectively, whereas corresponding specificity estimates were 83%, 90%, and 98%, respectively (Table 1; Fig. 2*D*). ROC areas ranged from 0.86 to 0.96 (Table 1).

It is possible that some hemophilia study subjects were in fact infected with HHV-8. To explore the effects of this possibility on our estimates of test specificity, we recalculated specificity for each test after excluding study subjects who were seropositive on at least two tests other than the test being evaluated. For the EIAs, we used cutpoints of 0.80 for the K8.1 EIA, 0.25 for both the orf73 and orf65 EIAs, and 0.10 for the whole virus EIA. In comparison with specificity estimates provided in Table 1, recalculated values increased by no more than 6%: specificity was 87% for the K8.1 EIA, 79% for the orf73 EIA, 88% for the orf65 EIA, 96% for the whole virus EIA, and 88% for the IFA.

Combinations of Tests

Combinations of individual tests, in which seropositivity was defined as a positive result for one or more tests in the combination, had increased sensitivity but decreased specificity, compared with each test used separately (Table 2). For example, the criterion "K8.1 OD > 1.50 *or* orf73 OD > 0.50" had 86% sensitivity and 94% specificity. Adding the orf65 EIA to this combination ("K8.1 OD > 1.50 *or* orf73 OD > 0.50 *or* orf65 OD > 0.50") further increased sensitivity (89%) but slightly decreased specificity (93%). Lowering the cutpoints for the individual EIAs also increased sensitivity and decreased specificity of combinations (Table 2).

Confirming all positive EIA results with IFA added specificity but lowered sensitivity, compared with not confirming EIAs (Table 2). For example, confirming individuals who were positive by the criterion "K8.1 OD > 1.50 *or* orf73 OD > 0.50" with IFA yielded more hemophilia study subjects who were judged HHV-8-seronegative (specificity increased from 94% to 98%) but

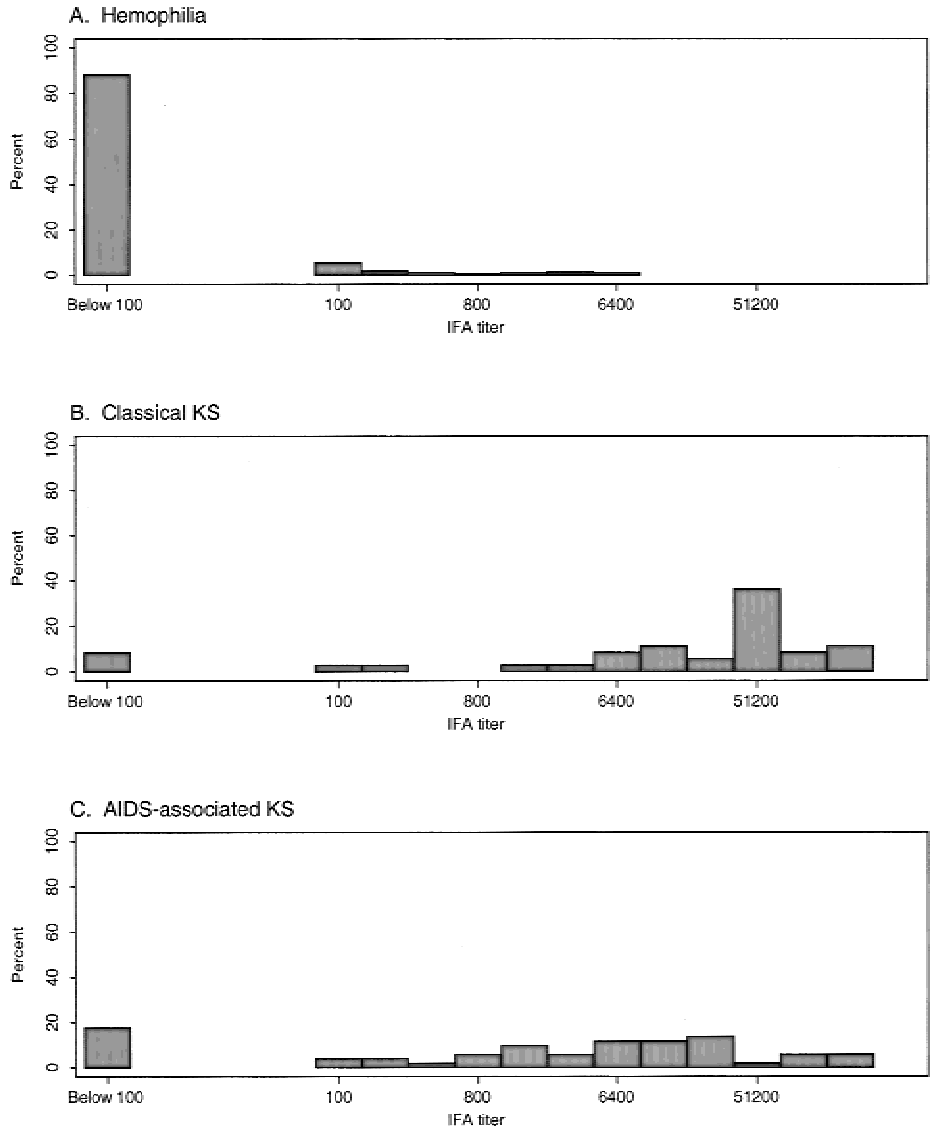

FIG. 1. Titers by indirect immunofluorescence assay (IFA) are shown for 210 hemophilic study subjects (**A**), 36 classical Kaposi's sarcoma (KS)-affected study subjects (**B**), and 51 AIDS-associated KS study subjects (**C**).

also fewer KS study subjects judged HHV-8 seropositive (sensitivity decreased from 86% to 80%).

We created classification trees to explore other assay combinations. In generating various candidate trees, we used only a derivation set of 224 study subjects. To create the tree presented in Figure 3, we restricted the choice of tests by the computer algorithm to only the K8.1 EIA and IFA, because this pairing included a test for lytic (K8.1) and latent (IFA) viral proteins, and because these tests individually performed well on diluted samples (see subsequent discussion). The tree in Figure 3 classified individuals as seronegative with K8.1 OD $\leq$ 0.50 and seropositive with K8.1 OD > 2.00. Individuals with K8.1 OD values between 0.51 and 2.00 were classified as seropositive or seronegative based on the IFA result. On the derivation set, this tree-based strategy had 90% sensitivity (95% confidence interval, 82%–97%) and 95% specificity (91%–98%). On the separate validation set of 73 study subjects (20 KS, 53 hemophilia), this strategy had 80% sensitivity (62%–98%) and 100% specificity (95%–100%; exact 95% confidence interval).

Sensitivity Using Diluted Samples

Individual tests and combinations of tests presented in Table 3 were 2% to 20% less sensitive in detecting antibodies in fourfold diluted KS samples than in undiluted KS samples. This loss in sensitivity was even more apparent for 16-fold dilutions. For example, using an OD cutpoint of 0.10 for the whole virus EIA, 84% of undi-

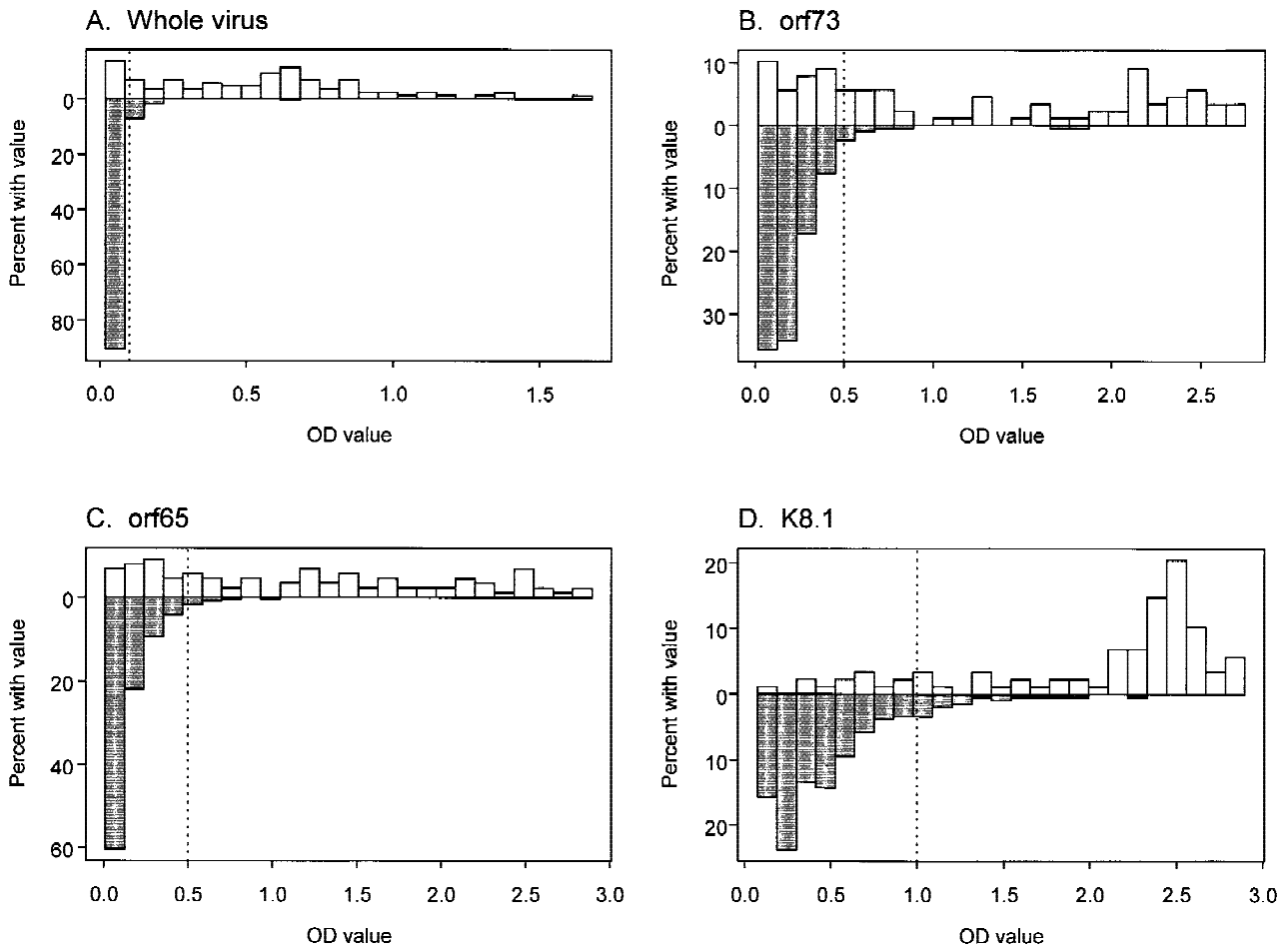

FIG. 2. Enzyme-linked immunoassay (EIA) results are shown for the whole virus (**A**), orf73 (**B**), orf65 (**C**), and K8.1 assays (**D**). In each panel, results are shown separately for study subjects with Kaposi's sarcoma (KS) (above the horizontal axis, *white bars*) and hemophilic study subjects (below the horizontal axis, *gray bars*). Examples of optical density cutpoints are shown, corresponding to cutpoints in Table 1: 0.10 (whole virus assay), 0.50 (orf73 and orf65 assays), and 1.00 (K8.1 assay). For each assay and cutpoint, sensitivity is the percentage of KS subjects with values above the cutpoint, and specificity is the percentage of hemophilic study subjects with values at or below the cutpoint.

luted KS samples were positive, whereas only 66% of fourfold-diluted and 45% of 16-fold–diluted KS samples were positive (Table 3). Losses in sensitivity were less pronounced for the K8.1 EIA (using a cutpoint of 0.80) and for IFA, with 73% and 75% sensitivity on 16-fold–diluted KS samples, respectively. Sensitivity using 16-fold–diluted samples remained at 65% or higher for several test combinations, including combinations of EIAs and the classification tree (Table 3).

DISCUSSION

Each evaluated serologic test, used by itself, discriminated at least moderately well between HHV-8-infected and HHV-8-uninfected individuals, with ROC areas greater than 0.85. Two assays (the whole virus and K8.1 EIAs) had high ROC areas (above 0.95). Nonetheless, because test values for infected and uninfected study subjects overlapped, we could not find cutpoints for any test that simultaneously provided sensitivity and specificity above 90%.

In contrast, several combinations of assays with appropriate cutpoints had better sensitivity and specificity. One of the best combinations of EIAs, "K8.1 OD > 1.50 *or* orf73 OD > 0.50 *or* orf65 OD > 0.50," had 89% sensitivity and 93% specificity. Some strategies incorporating IFA confirmation of EIA-positive samples also performed well (Table 2). IFA itself, however, was only 86% sensitive, so overall sensitivity was limited for strategies that used IFA confirmation of all EIA-positive specimens.

The classification tree depicted in Figure 3 has advan-

TABLE 1. *Performance of individual tests*

Test	Cutpoint, OD value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	ROC area (94% CI)
EIAs				
K8.1	0.80	90 (83–96)	83 (78–88)	0.96 (0.93–0.99)
	1.00	85 (78–93)	90 (85–94)	
	1.50	78 (69–87)	98 (96–100)	
orf73	0.25	84 (76–92)	73 (67–79)	0.86 (0.81–0.91)
	0.40	72 (63–82)	93 (89–96)	
	0.50	68 (58–78)	97 (94–99)	
orf65	0.25	86 (79–93)	82 (77–88)	0.93 (0.89–0.97)
	0.40	75 (66–84)	95 (92–98)	
	0.50	70 (60–80)	98 (96–100)	
Whole virus	0.10	84 (76–92)	95 (92–98)	0.96 (0.93–0.99)
	0.15	80 (72–89)	98 (96–100)	
	3 × negative controls[a]	77 (68–86)	99 (98–100)	
IFA	—	86 (79–93)	88 (83–92)	0.87 (0.82–0.92)

[a] For this row of the table, the OD cutpoint for the whole virus EIA was three times the mean OD value of negative controls on the same plate as the samples (per manufacturer's instructions).

OD, optical density; CI, confidence interval; ROC, receiver operator characteristic; EIAs, enzyme immunoassays; IFA, immunofluorescence assay.

tages over other testing strategies. Individuals were considered HHV-8-seropositive if they had a K8.1 OD > 2.00 or both an intermediate K8.1 result (OD, 0.51–2.00) *and* a positive IFA. The tree-based strategy thus used IFA only to interpret indeterminate K8.1 results, not to confirm all K8.1 results. We expected that this strategy would perform very well on the derivation set, because a highly flexible algorithm fitted the tree directly to these data. In addition, on the separate validation set, specificity remained very high (100%); sensitivity may have been somewhat lower (80%), but with relatively few KS study subjects, the 95% confidence limits were wide (62%–98%). The tree-based test strategy would be efficient for testing large sample collections, because the labor-intensive IFA would only be needed for the subset of study subjects with intermediate K8.1 OD values. With this strategy, the number of IFAs performed would depend on the prevalence of infection in the population.

TABLE 2. *Performance of combinations of tests*

Combination of tests	Cutpoint, OD value				Sensitivity, % (95% CI)	Specificity, % (95% CI)
	K8.1	orf73	orf65	WV		
EIAs used without IFA confirmation						
K8.1 or orf73	0.80	0.25	—	—	95 (91–100)	64 (57–70)
	1.00	0.40	—	—	91 (85–97)	84 (79–89)
	1.50	0.50	—	—	86 (79–93)	94 (91–97)
K8.1 or orf73 or orf65	0.80	0.25	0.25	—	98 (95–100)	60 (53–67)
	1.00	0.40	0.40	—	93 (88–98)	82 (77–87)
	1.50	0.50	0.50	—	89 (82–95)	93 (90–97)
K8.1 or orf73 or orf65 or whole virus	0.80	0.25	0.25	0.10	98 (95–100)	59 (52–65)
	1.00	0.40	0.40	0.15	93 (88–98)	80 (75–86)
	1.50	0.50	0.50	3×[a]	89 (82–95)	93 (89–96)
IFA confirmation of positive EIA results						
K8.1 or orf73	0.80	0.25	—	—	84 (76–92)	95 (92–98)
	1.00	0.40	—	—	82 (73–90)	97 (95–99)
	1.50	0.50	—	—	80 (72–89)	98 (96–100)
K8.1 or orf73 or orf65	0.80	0.25	0.25	—	86 (79–93)	94 (91–97)
	1.00	0.40	0.40	—	83 (75–91)	96 (94–99)
	1.50	0.50	0.50	—	82 (73–90)	98 (96–100)
K8.1 or orf73 or orf65 or whole virus	0.80	0.25	0.25	0.10	86 (79–93)	94 (91–97)
	1.00	0.40	0.40	0.15	83 (75–91)	96 (93–98)
	1.50	0.50	0.50	3×[a]	82 (73–90)	98 (96–100)

[a] For this row of the table, the OD cutpoint for the whole virus EIA was three times the mean OD value of negative controls on the same plate as the samples.

OD, optical density; WV, whole virus; CI, confidence interval; EIA, enzyme-linked immunoassay; IFA, immunofluorescence assay.

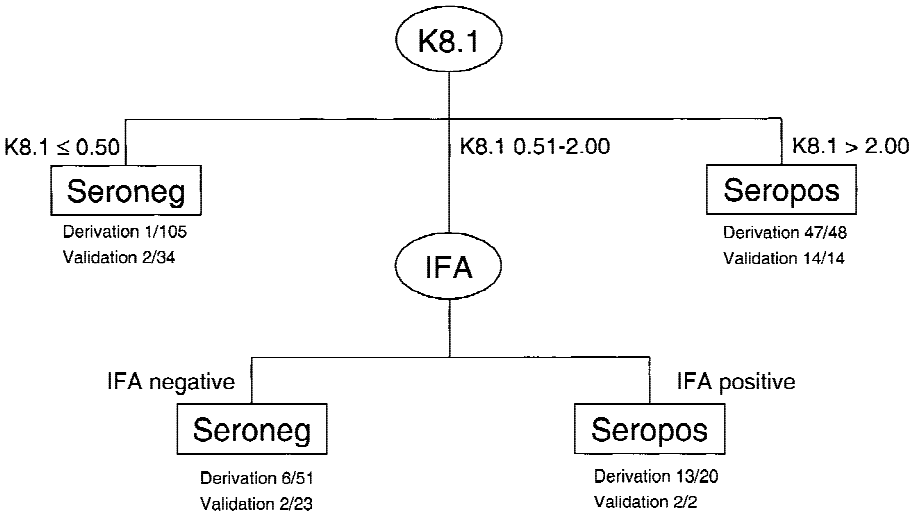

FIG. 3. Displayed is a classification tree that identifies study subjects' human herpesvirus 8 serostatus. In the tree, branch points (K8.1 enzyme immunoassay and immunofluorescence assay [IFA] testing) are indicated by *ellipses,* whereas terminal nodes (where individuals are classified as seropositive or seronegative) are indicated by *rectangles.* The tree classifies as seropositive individuals with K8.1 optical density (OD) > 2.00 or with both an intermediate K8.1 OD (0.51–2.00) and positive IFA test result. Below each terminal node is the number of study subjects at that node with Kaposi's sarcoma over the total number of study subjects at that node, for the derivation dataset (*N* = 224) and validation dataset (*N* = 73).

In our sample, intermediate K8.1 results were found in 23 KS study subjects (26%) and 73 hemophilia study subjects (35%). With refinements in the K8.1 EIA, a test still under development, fewer persons might have indeterminate K8.1 results.

Specificity of serologic assays has been poorly characterized, partly because it is difficult to identify a population known to be HHV-8 uninfected. For this purpose, we chose men with hemophilia and their partners, a group with low KS risk (12). Nonetheless, for each assay and cutpoint, some individuals were seropositive, which we interpreted as false positive results. Such false positive results could be due to measurement error, cross-reacting antibodies to other herpesviruses, or nonspecific antibodies. It is possible that a few hemophilia study subjects were truly HHV-8-infected, leading us to underestimate test specificity. However, specificity did not improve substantially when we excluded hemophilia study subjects who were positive on more than one test, suggesting that our specificity estimates were accurate.

No tests or test combinations with at least 90% specificity identified all individuals with KS, suggesting that some immunocompromised people do not make detectable HHV-8 antibodies. Our estimates of sensitivity for some tests are lower than others have found (6,18), but direct comparisons across studies are complicated by differences in laboratory methods. For all assays, evaluating test sensitivity for asymptomatic infection is problematic, given that asymptomatic people lack an identifiable illness and thus are not easily studied. When we measured IFA titers of our hemophilia study subjects, we noted that the few people who were IFA seropositive had

TABLE 3. *Sensitivity of assays using diluted samples*

Tests/combinations of tests	Cutpoint, OD value				Sensitivity %	Sensitivity on 4-fold diluted KS, %	Sensitivity on 16-fold diluted KS, %	Specificity, %
	K8.1	orf73	orf65	WV				
EIAs								
K8.1	0.80	—	—	—	90	82	73	83
orf73	—	0.25	—	—	84	64	53	73
orf65	—	—	0.25	—	86	79	56	82
Whole virus	—	—	—	0.10	84	66	45	95
IFA	—	—	—	—	86	79	75	88
EIAs without confirmation								
K8.1 or orf73	1.50	0.50	—	—	86	81	65	94
K8.1 or orf73 or orf65	1.50	0.50	0.50	—	89	87	72	93
EIAs with IFA confirmation								
K8.1 or orf73 or orf65	0.80	0.25	0.25	—	86	76	69	94
Classification tree								
Development set	(see Fig. 3)				90	82	72	95
Validation set	(see Fig. 3)				80	80	65	100

OD, optical density; WV, whole virus; KS, Kaposi's sarcoma subjects; EIA, enzyme-linked immunoassays; IFA, immunofluorescence assay.

substantially lower titers than KS study subjects (Fig. 1). If some of these seropositive hemophilia study subjects were truly infected, then our data (and similar results from other studies [5,6]) imply that asymptomatically infected individuals do indeed have lower antibody levels than KS study subjects.

To model detection of asymptomatic HHV-8 infection, we applied our assays to diluted samples from KS study subjects. Assays and combinations of assays with at least 90% specificity were able to detect 66% to 87% of fourfold diluted specimens, but only 45% to 72% of 16-fold diluted specimens (Table 3). The orf65, orf73, and whole virus EIAs exhibited large drops in sensitivity on diluted samples, whereas the K8.1 EIA and IFA retained reasonable sensitivity. By using diluted samples, we examined only quantitative differences in antibodies between individuals with KS and those with asymptomatic infection. In reality, qualitative differences might also be important. Nonetheless, because most HHV-8-infected people are asymptomatic, this analysis demonstrates that overall assay sensitivity is lower than can be appreciated by examining only patients with KS.

Despite shortcomings, most evaluated test strategies probably perform well enough for epidemiologic studies that examine relative differences in HHV-8 infection rates between groups. Group differences can often be identified despite some misclassification of individuals. However, HHV-8 seroprevalence estimates, measured in absolute terms, depend strongly on test performance. For low-risk populations, small changes in assay specificity have a large effect on seroprevalence estimates, and even highly specific tests overestimate the proportion of individuals infected. For example, if 5% of a low-risk population (such as blood donors) is truly HHV-8-infected, apparent seroprevalence can range from 9% to 23%, using tests with sensitivity and specificity similar to those of assays that we examined (Table 4). Unfortunately, interpretation of published seroprevalence estimates remains difficult, because sensitivity and specificity of the various HHV-8 serologic tests are not known with certainty.

Of importance, many seropositive individuals in low-risk populations are actually uninfected, because positive predictive values of HHV-8 tests are no higher than 50% (Table 4). These low values limit application of serologic tests outside research settings. For instance, HHV-8 can be transmitted through solid organ transplantation (24), but a screening program that used available tests would have dubious value in low prevalence countries, because relatively few seropositive donors or recipients would be infected. Finally, because few seropositive individuals are actually infected, slight differences among tests in the individuals identified as seropositive may easily arise and lead to discordant assay results (4).

TABLE 4. *Seroprevalence estimates for a hypothetical population with 5% of individuals infected with human herpesvirus-8 (HHV-8)*[a]

Sensitivity, %	Specificity, %	Seroprevalence, %	Positive predictive value, %
95	95	10	50
95	85	19	25
95	80	24	20
85	95	9	47
85	85	19	23
85	80	23	18
80	95	9	46
80	85	18	22
80	80	23	17

[a] The table provides seroprevalence estimates and positive predictive values for a hypothetical population in which the true prevalence of HHV8 infection R is 5%. For different values of sensitivity and specificity, seroprevalence is calculated from the formula $S = [R \times \text{sensitivity} + (100 - R) \times (100 - \text{specificity})]/100$. Positive predictive value is $(R \times \text{sensitivity})/S$.

Our results should be applied cautiously for regions outside North America and Europe. Studies in sub-Saharan Africa generally reveal high seroprevalence, in agreement with high KS rates. Nonetheless, assays may have lower specificity in this setting than we observed, perhaps due to the presence of other infections. For example, high seroprevalence in West Africa (25,26), where KS has been rare (27), may be due to low assay specificity rather than high infection rates.

Several avenues of investigation might improve HHV-8 serologic tests and understanding of their performance. Our results should be replicated in other groups likely to be infected (e.g., asymptomatic individuals who later develop KS) or uninfected (e.g., children from countries where KS is rare). Additionally, future investigations might clarify the status of asymptomatic individuals with positive serology, perhaps through longitudinal studies. If asymptomatic individuals with low antibody levels are truly infected, then assay sensitivity estimates should be lowered substantially. Finally, new assays and refinements of existing assays may better identify HHV-8 infection. Improved assays must be assessed rigorously using carefully characterized samples from infected and uninfected individuals.

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